Bioprospecting Potential Probiotics from Human Gut Microbiome of Rourkela Population, Odisha

Thesis Submitted to National Institute of Technology, Rourkela in Partial Fulfilment of the Requirements for the Degree of

MASTER OF SCIENCE

In Life Science

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DECLARATION

I do hereby declare that the Project Work entitled "***Bioprospecting Potential Probiotics from Human Gut Microbiome of Rourkela Population, Odisha***", submitted to Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science, is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Rasu Jayabalan, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

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Place: Rourkela

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CERTIFICATE

This is to certify that the thesis entitled "***Bioprospecting Potential Probiotics from Human Gut Microbiome of Rourkela Population, Odisha***" which is being submitted by **Mr. Kumar SagarJaiswal,** Roll No. **413ls2032**, for the degree of master of science in Life Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institute for the award of any degree or diploma.

R. Jayabalan 11/5/15

R.Jayabalan

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Kumar Sagar Jaiswal

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List of Abbreviations

Gm	**Gram**
µL	**Microlitre**
ml	**Millilitre**
min	**Minute**
%	**Percentage**
^{O}C	**Degree Celsius**
MRSA	**de Man Rogosa Sharpe Agar**
Spp.	**Species**
mm	**Milimeter**
cm	**Centimeter**
SGF	**Simulated Gastric Fluid**
GIT	**Gastro Intestinal Tract**
CFU	**Colony Forming Unit**

Abstract

Probiotics are live microorganisms known to be conferring health promoting effects on their hosts when consumed in sufficient amount. There are lots of sources are available for potent probiotic strain but strains isolated from human origin are more preferred because microorganisms isolated from human sources will have more adaptability to live in human gut than from non-human sources.

Faecal material of a human being contains various excretory substances from gastrointestinal tract. This also includes the microorganisms from gut microbiota. As probiotics are important inhabitants of gut, hence there is a high chance of getting more and indigenous probiotic strains.

Isolates used in this study are verified and identified by various biochemical, pre-determined probiotic properties and lastly by genotypic identification. Four best isolates were chosen and tested for various properties such as acid and bile tolerance, cell surface hydrophobicity, antimicrobial activity against food-borne pathogens, two additional properties i.e. cholesterol reduction and ACE inhibitory effects were also tested. In many tests our isolates showed best results than the *L. casei Shirota* of commercial fame.

Molecular and genotypic identification through 16S rDNA sequencing confirmed that out of four isolates one is *Lactobacillus plantarum* and another one is *Weisella confusa*. In this it was observed that human faecal material is also a potent and successful source of probiotic strains.

Keywords:- Probiotics; *Lactobacillus plantarum; Weisella confusa*; Antimicrobial activity.

1 Introduction

1.1 Probiotics

“Probiotics” a term derived from the conjugation of two different Greek words, which are “Pro” means “for” and “bios” means for “life”. So the literary meaning of this term defines itself as “essential for life”. Probiotics has become a great topic of interest and research throughout the last decade. Elie Metchnikoff, a famous biologist was the first to find out the importance of the intestinal microbiota in maintaining the homeostasis of human body and longevity (Metchnikoff, 1907). In 1965, Lilly and Stillwell coined this term “probiotics” referring as “microbially derived factors that stimulate the growth of other organisms” (Guarner et al., 2008). As a term “probiotic” became popular by R. Fuller, and this was defined as “a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance” (Fuller, 1989). This definition was later extended to include other beneficial effects such as immunomodulation. Probiotics are: “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” and this has been defined by FAO/WHO organization in 2001. As well as in Italy, the Ministry of Health has also provided a definition for probiotics as “microorganisms which, once ingested in adequate amounts, have beneficial effects on the organism”.

1.2 Human gut microbiota

The human gastrointestinal tract is colonized with a differing populace of microbial verdure that gives digestive capacity as well as additionally contributes towards intestinal epithelial homeostasis and innate immunity. Any type of alteration in the intestinal microflora has been ensnared in the pathogenesis of different diseases which include infection, allergy, inflammation and serious immunological conditions. One can also define the intestinal microbiota as an ecosystem which is composed of several types of ecological niche. Then these niches are further containing various bacterial species as well as huge variety of strains. The intestinal mucosa and microbiota remain in close corporation acquiring largest surface area of the body (Aureli et al., 2011). A mucosal boundary has been formed by this corporation which acts as an active defence mechanism in opposition of potential pathogen and immunogens present in the lumen. Just after the birth, when the human infant is subjected to breast feeding a complex microbiota starts developing in gastrointestinal tracts. Initially this ecosystem is dominated by Bifidobacteria, but as the infant grows the intestinal environment gets exposure to various factors and another microbiota start developing by

reducing the population of Bifidobacteria (Embleton and Yates, 2008). In human intestine about 100 trillion bacterial cells from 400 diverse species are found which is too much high when compared to the population of host cells (Bäckhed et al., 2005). The homeostasis of the gut is always altering throughout the life and main responsible factors are environmental pollution and other stress conditions, which ultimately leads to some acute and chronic disorders. These condition leads decrease in the population of beneficial microbes such as Lactobacilli and Bifidobacteria to conceivably unsafe pathogens of Clostridia, Sulphate reducers and Bacteroides species. Prevalence of these microbes in the gut makes the body more susceptible to diseases. Therefore to lower down the adverse effects arising in gut, probiotics are the best option available and their administration will be very helpful for restoring the homeostasis of GIT (Sathyabama et al., 2012). Probiotics have been used to protect the host from the various intestinal diseases and the increase in population of these health promoting bacteria bring the homeostasis (Fooks et al., 1999). From the ancient times probiotics has been taken as an food supplement to balance homeostasis of intestinal microbiota (Holzapfel et al., 1998). The probiotics are being more favourable than the other microbes because these are naturally found in the intestinal tract of human (Çakır, 2003).

1.3Characteristics of probiotics

Certain characteristics have been defined for a microorganism to be called as probiotics as well as to be effective enough. Some of the important characteristics are tolerance to gastric juice and bile, adherence towards intestinal mucosa, and most of all antimicrobial activity against pathogens (Collins et al., 1998). Characteristics of a successful probiotics as follows

(1) Have a demonstrated beneficial effect on the host.

(2) Be non-pathogenic, non-toxic and free of significant adverse side effects.

(3) Be able to survive through the gastrointestinal tract (GIT; in vitro and in vivo).

(4) Be present in the product in an adequate number of viable cells to confer the health benefit.

(5) Be compatible with product matrix, processing and storage conditions to maintain the desired properties, and labelled accurately (Collado et al., 2010).

1.4 Sources for probiotics

Dairy and dairy based product are rich source of probiotics (Liong, 2011). Spontaneous milk fermentation and use of these fermented products has been in use for centuries and mostly the source of lactic acid bacteria (LAB), bifidobacteria and other microbes for human use. Isolation of probiotics from human faecal samples are also in practice, which gives a better result because the bacteria existing in the gut of human being. Table 1 shows different probiotic bacteria for human uses.

Table 1. Microorganisms applied in probiotic products

Lactobacillus species	*Bifidobacterium Species*	*Others*
L. acidophilus	*B. bifidum*	*Leuoconostoc mesenteroides*
L. rhamnosus	*B. animalis*	*Enterococcus faecium*
L. gasseri	*B. breve*	*Streptococcus salivarus subsp. thermophilus*
L. casei	*B. infantis*	*Lactococcus lactis subsp.lactis*
L. crispatus	*B. longum*	*Lactococcus lactis subsp.cremoris*
L. delbrueckii subsp. bulgaricus	*B. lactis*	*Propionibacterium freudenreichii*
L. reuteri	*B. adolascentis*	*Pediococcus acidilactici*
L. helveticus		*Enterococcus faecalis*
L. fermentum		*Saccharomyces boulardii*
L.plantarum		
L. gallinarum		
L. johnsonii		
L. plantarum		
L. salivarus		

1.4 Industrialization and Commercialization of probiotics

Now-a-days probiotics has become of high industrial and commercial value. They are now being used in various fields which has a direct or indirect effect on human health. These are available as drinks, yogurt, tablets, capsules and some fermented foods, which contain these probiotics in adequate amount so that they confer a health promoting effect on the consumer. These homemade as well as commercialized products can contain one or several species of probiotic bacteria. The products which are in use for human health are available as fermented milk, tablets, capsules but these capsules or tablets have not been used for medical applications. Their simple principle is to promote the gut microbial flora and eradicate the harmful effects of microbial disorders in the gut. Commercial probiotic products are generally available either as diary based products or non-diary based products (Hamilton-Miller et al., 1999). Diary based food products include yogurt, curd, kefir, cheese etc. whereas the non-diary food products include fermented vegetable juices, fermented fruits and berry juices, probiotics salami and probiotic olives etc.

2. Review of Literature

2.1 Effects of probiotics on health

Till now lots of studies have been accomplished on the effects of probiotics on health but those test subjects were sufficient or the microorganisms have not been identified (Çakır, 2003). Some effects have been partially established but more of them are now clinically well verified. Some of the beneficial effects have been documented below (Schrezenmeir and de Vrese, 2001). Some of the clinically proven effects of probiotics have been presented in figure 1.

- Digestion of lactose in lactose intolerant.
- Betterment of immune system.
- Reduction of risk of colon cancer.
- Lowering the level of cholesterol and triacylglycerol in blood plasma.
- Management of blood pressure.
- Bringing down inflammation.
- Diminishing allergic symptoms.
- Advantageous impacts on metabolism of minerals, preferably bone volume and stability.
- Diminishing effects on infection of *Helicobacter pylori*.
- Exclusion of pathogens (antimicrobial effect).
- Osteoporosis avoidance.
- Lesser risks of urogenital infections.

2.1.1 Lactose Intolerance

Lactose intolerance has become a digestive problem in several population of whole Europe. People with this defect are unable to digest lactose present in the food products because of absence of enzyme β-galactosidase which breaks down the lactose to glucose and galactose. Whenever these lactose intolerant people consume milk or other food products containing lactose they show symptoms of abdominal pain, bloating, flatulence and cramping. As lactose passes through the small intestine the colonic microflora form gas and in the large intestine its get converted to acid. Incomplete digestion of lactose also can be known by the presence

of hydrogen in breath. Certain probiotic starter cultures are when added to milk and other food products and when these are consumed by lactose intolerant people, they generally don't show any symptoms of lactose intolerance as well as no rise in level of breath hydrogen (Fooks et al., 1999).

There are two possible explanation for the beneficial effect of probiotics on lactose intolerance. First one is that, it has been found that the concentration of lactose in fermented foods are very low and this is only due to the lactase activity of probiotic bacteria present in that food or used for production of that food. Second explanation is that when these fermented foods are consumed then active lactase enzyme and probiotic bacteria with increased lactase activity enter the human gut (Salminen and Von Wright, 2004). Yogurt in comparison with milk, more preferred to be consumed by lactose intolerant people because the lactose has been converted into lactic acid and it also have those bacterial species which produce β-galactosidase (Salminen and Von Wright, 2004). Predominantly *L.bulgaricus* and *Streptococcus thermophilus* are used for the production of yogurt and studies show that these strains have no resistance to gastric acidity, this simply leads to conclusion that food products with probiotics should be more preferred to lactose intolerant people.

The digestion of lactose is not only dependant on bacterial β-galactosidase, but also another factor is the slow gastric emptying of semi-solid milk products such as yogurt in the stomach (Salminen and Von Wright, 2004).

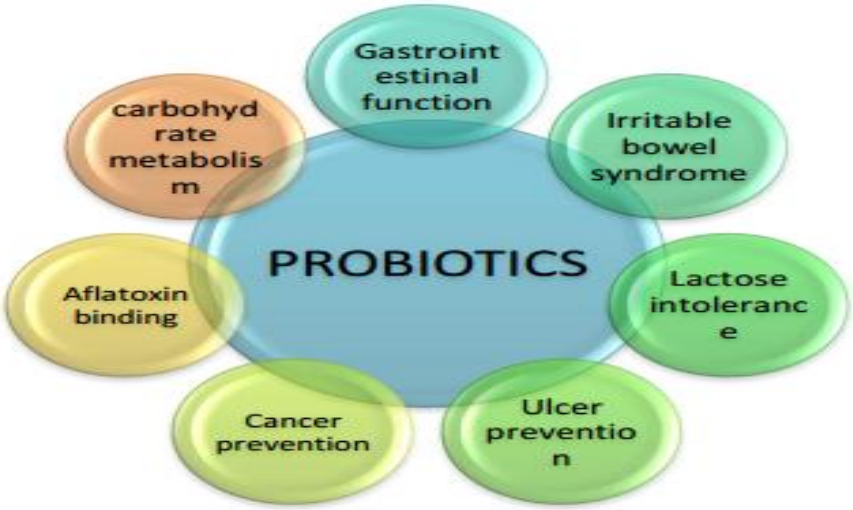

Figure 1. Diagrammatic representation of proven clinical benefits of probiotics

2.1.2 Probiotics and Immune system

Probiotics have shown a promising effects on immune system but the mechanism of action is under study till now. Probiotics have shown positive results that they have a health promoting effect on host when tested on human subjects (Mombelli and Gismondo, 2000). Studies have been carried out to know effects of probiotics on human system with both in-vitro and in-vivo conditions in mice as well as in human. These studies reveal that when probiotic bacteria has been taken in mode of oral bacteriotherapy, they have promising effects on immune system as well as they show antagonistic activity on some pathogens (Scheinbach, 1998) and (Dugas et al., 1999).

The effect of probiotics on immune system can be of different types. Among which production of cytokines, activation and stimulation of macrophages and last but not least they are capable enough to increase the concentration of secretory IgA (Çakır,2003; Scheinbach,1998; Dugas, et al. 1999). Adhesion of these microorganisms is the main cause for some the effects.

The study of Link-Amster et al. (1994) shows the immunomodulatory effect of probiotics. In his study he tested whether consumption of fermented milk with *L. acidophilus La1* and *bifidobacteria* are capable of bringing any effect in human. In the test human volunteers were provided with fermented milk for three weeks as well as attenuated *Salmonella typhi* Ty21a was administered into them to mimic the pathogenic infection. After three weeks it was found that specific serum IgA level has increased to 4 fold in comparison of the control group which only administered the attenuated *Salmonella* infection. The conclusion of this study was that the LAB strains which can survive in the acidic conditions of GIT, are capable of act as adjuvants to the humoral immunity response (LimeAmster et a, 1994, Quwehand et al,1999). In the study of Perdigon et al (1986), where he fed the mice yogurt containing lactobacilii and found the stimulation of macrophages and increased level of IgA concentration (Scheinbach,1998). Halpern et al. (1991) commenced a human trial in which 450g of yogurt per day was given continuously for four months and a significant increase found in production of γ-interferon (Fooks et al, 1999). In vitro suppression of lymphocyte proliferation by extracts of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis*Bb-12 was studied by Mattilla-Sandholm and Kauppila (1998).

2.1.3 Diarrhoea

Even if diarrhoea is caused by many agents and there are many types of it, there are studies which tried to evaluate the effects of probiotics on it. One among the several reasons for children death in world is diarrhoea and its main causative agent is rotavirus (Scheinbach,1998). *Lactobacillus GG* has found to be most effective against rotavirus diarrhoea as it has the capability to lower down the infection effect of the rotavirus and this has been proved by studies of many researchers. Other probiotic bacteria such as *Lactobacillus acidophilus* LB1, *Bifidobacterium lactis* and *Lactobacillus reuterii* have been found to have profound effect on diarrhoea (Salminen et al. 2004).

Another type of diarrhoea is the Traveller's Diarrhoea (TD), effecting the travellers among the whole world. In a study, done by Oksanen et al. (1990), it has been found that *Lactobacillus GG* is capable enough for preventing TD. Another study of Black et al (1989), where he used lyophilised form of several bacteria such as *L.acidophilus, B.bifidum, L.bulgaricus, S.thermophilus,* were given to travellers and the occurrence of TD was observed and it was found that the group which has taken those lyophilised bacteria showed TD in 43% of cases and the control group which didn't receive those bacteria showed 71% of TD (Gismondo et al 1999).

Antibiotic associated diarrhoea (AAD), is also one type of diarrhoea which is a result of severe antibiotic therapy which causes imbalance in the gut microbiota homeostasis. Among which *Clostridium difficile* are the resistant strains arise due to antibiotic therapy and causes AAD. *Saccharomyces boulardii, Lactobacillus spp.* and *Bifidobacterium spp.* have been used for several clinical trials and showed promising effects. Many studies reveal that use of *Saccharomyces boulardii* during AAD is most effective in eradicating the population of *Clostridium difficile* from gut microflora (Gismondo et al. 1999).

2.1.4 Effects against Cancer

There are several bacterial enzymes which are responsible for converting the precarcinogens into carcinogens in the colon, and these enzymes include β- glucuronidase, nitroreductase and azoreductase. It has been thought that probiotics have the capability of reducing the activity of these bacterial enzymes but the exact mechanism is not known till now. But there are few explanation have been made about the action of probiotics which have been proposed by McIntosh as follows (Fooks, et al. 1999; Scheinbach, 1998):-

1. Deactivation of Carcinogens or precarcinogens by attaching and blocking of their active sites.

2. Exclusion of the bacteria producing the enzymes required for activation of procarcinogens to carcinogens.

3. The pH of intestine changes which alters the pathogenic activity.

4. Colonic emptying time change leads to sufficient removal of harmful mutagens through faeces.

5. Activation of immune system.

The studies from both animal as well as human have shown that the occurrence of DNA damage in colon due to chemical carcinogens have been lowered due to regular oral administration of LAB. When fermented food products and milk products housing lactobacillus and bifidobacteria are consumed in a large quantity there is a low chance of colon cancer (Hirayama and Rafter, 2000).

2.1.5 Reduction of Cholesterol

Cholesterol lowering effects of probiotics has well set now but the mechanism of action in unknown till now. Two possible explanation are there for this and first one explains that bacteria has capability of binding to the cholesterol molecule directly into the cell membrane. Second explanation is, there are several bile salt hydrolysis enzymes which break down the increased level of cholesterol (Prakash and Jones, 2005).

A study on mice where it has been fed by *Lactobacillus reuteri* CLR1098 for 7 days around 10^4 cells per day show reduction of cholesterol by 38%. The same dose of this probiotic resulted in lowering of triglycerides by 40% and increase of 20% in the ratio of high density lipoprotein to low density lipoprotein (Kaur et al., 2002).

2.2 Mechanism of action of probiotics

There are several mechanisms which explain the mode of action of probiotics and some of them have been explained here briefly (Rolfe, 2000) :-

1. Inhibitor production:- Probiotics produce organic acids, bacteriocins and hydrogen peroxide which act as inhibitors for both Gram-positive as well as Gram-negative microbes.
2. Adhesion site blockage:- Probiotics act as competitive inhibitor against the pathogens to bind to the intestinal epithelium.
3. Competition for resources:- Probiotics inhibit the pathogen growth in the gut by competing for nutrients needed for survivability.
4. Immunomodulatory effect:- The immunologic benefits provided by probiotics are prevention of allergies is due to activation of macrophages that increase the antigen presentation to B lymphocytes and increases secretion of IgA.
5. Toxin receptor degradation:- The toxin receptor sites of intestinal mucosa are degraded by the action of probiotics. One of the best example of this mechanism is inhibition of *C. difficile* infection by *S. boulardii*.

Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence (Fooks et al, 1999). Figure 2 summarizes the mechanism of action of probiotics.

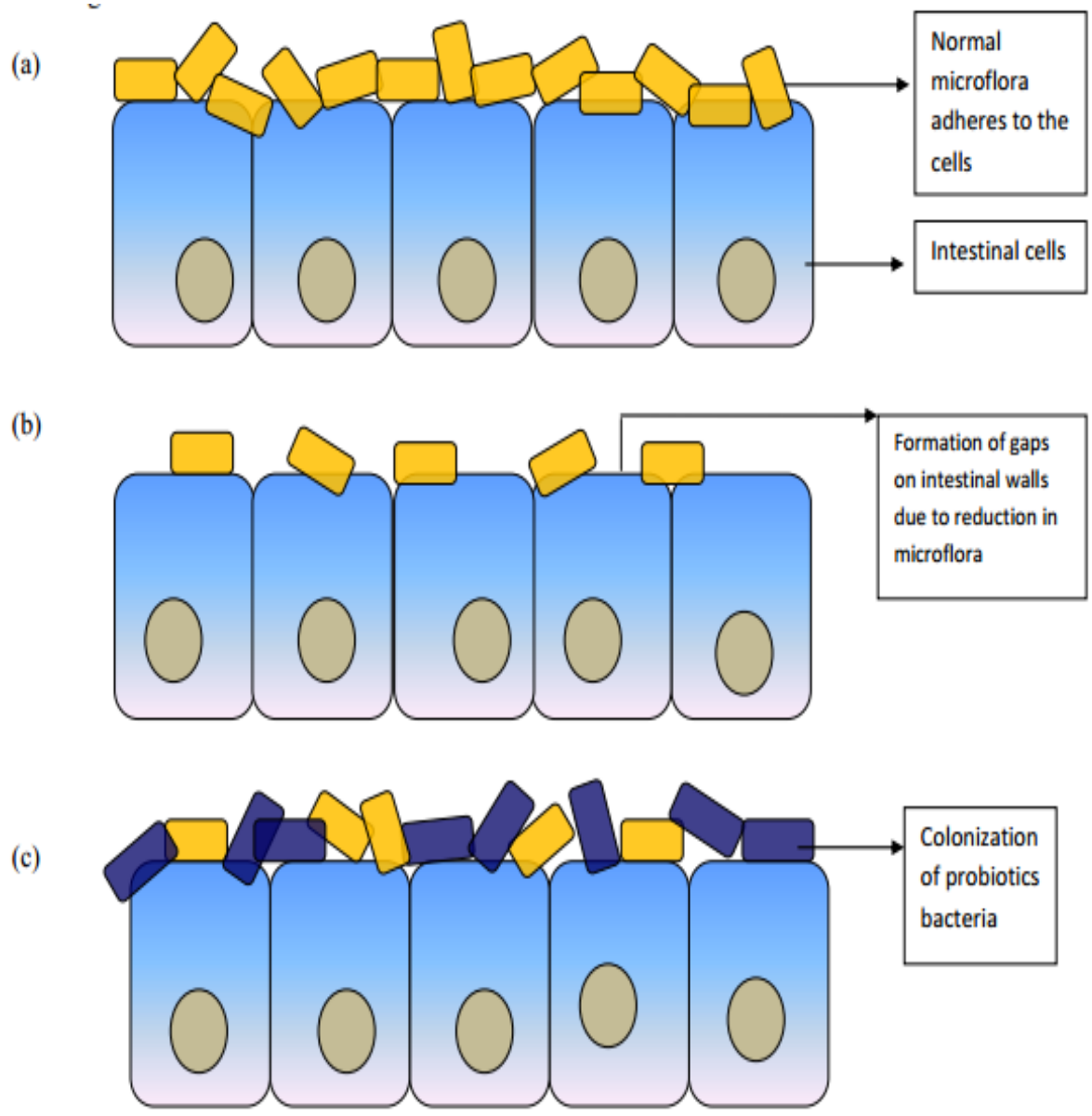

Fig 2. Role of probiotics in inhibition of growth of pathogenic bacteria.

(a) Intestinal epithelia has been covered up by normal microbiota shown in yellow. (b) Disturbance in homeostasis of normal microbiota leaves space for growth and adherence of pathogens. (c) Use of probiotics (blue) causes leads to their adherence on intestinal epithelia which diminishes the chance of survivability of pathogens.

2.3 Isolation, identification, characterisation and safety of probiotics

More and more and new potential probiotics discovery for use is not an easy task. The methods employed must provide essential data and information concerning microbial ecosystems, including the sources of probiotics. The very first also important step in studying a specific microbial ecosystem is isolation of its members. If it is going to be probiotic then molecular identification must be done. After identification there are several tests for characterizing these bacteria are followed. At last the safety assessment should be done. Figure 3 gives a brief idea about these whole processes.

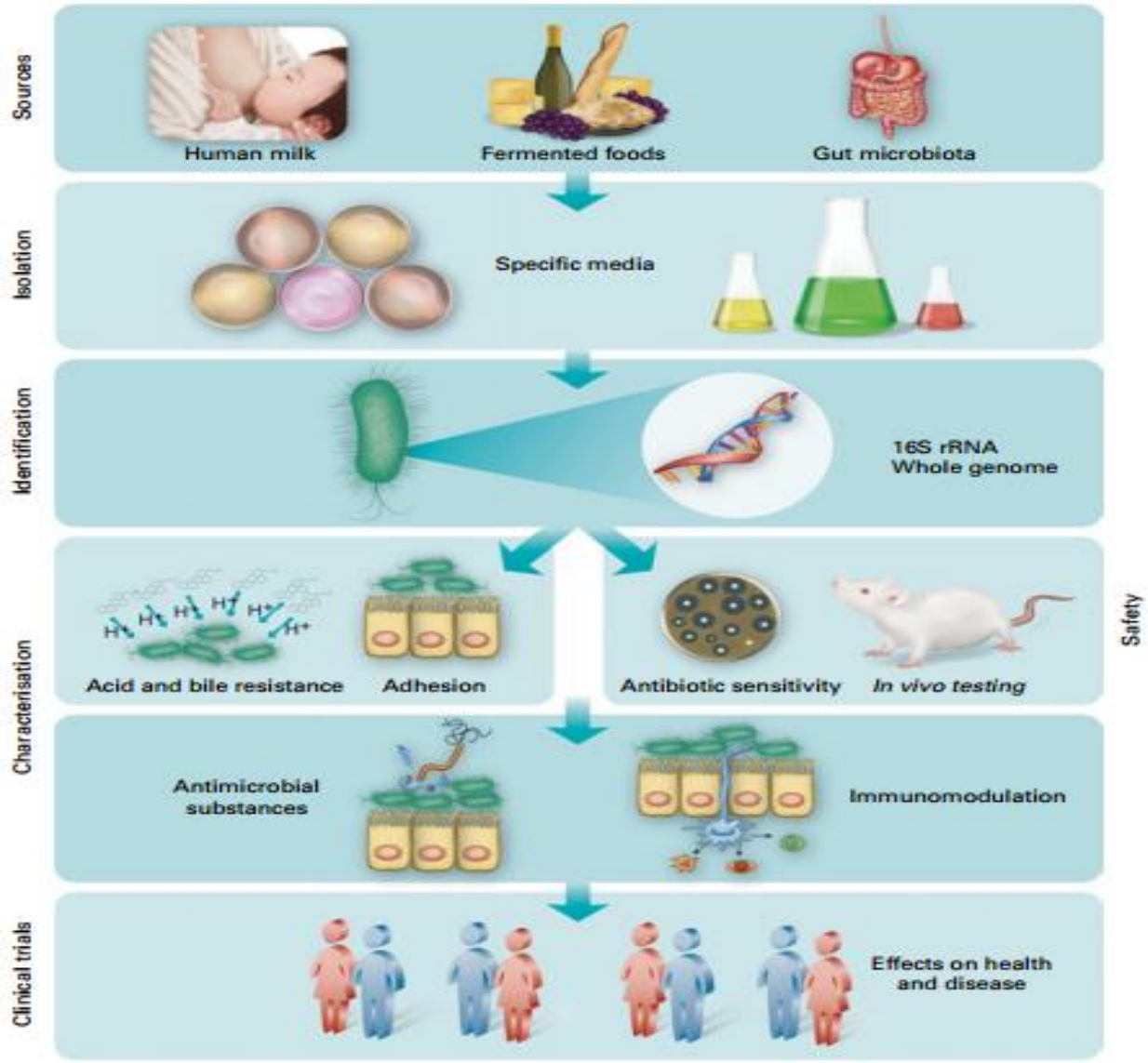

Fig 3:- Flow chart describing the various steps to be followed in order for a bacterial strain to qualify as a novel probiotic.

2.3.1 Sources of isolation

Till now traditional fermented dairy based food products have been used as a source of isolation because they contain diverse compositions of LAB species. In a recent study 148 LAB strains have been isolated from "Kurut" a natural fermented yak milk product in China, predominantly having populations of *L. delbrueckii subsp. bulgaricus* and *Streptococcus thermophiles.* Additionally several yeasts and *Lactobacillus* strains having ability to influence immune response have been also isolated from Kefir grains (Lopitz-Otsoa et al., 2006), Masai milk (Patrignani et al., 2006) and Koumiss a fermented milk drink (Ya et al., 2008). For evaluation of traditional fermented products as potent source for probiotics show that the isolated microorganisms belong to *Lactobacillus* genus (Lim and Im, 2009), (Won et al., 2011). Recently from a Nigerian fermented food product *Weisella* strain have been isolated and tested for its probiotic characteristics (Ayeni et al., 2011).

Cheese a fermented diary product is the most potent as well as best source for delivery of probiotics into the human intestine. *L. plantarum* strains have been predominantly isolated from Italian, Argentinean (Ugarte et al., 2006) and Bulgarian cheese (Zago et al., 2011).The non-sterile breast milk collected aseptically and studied, it has been found that breast milk contain the natural probiotic inoculum (West et al., 1979). The LAB strains isolated from breast milk were also found to be present in the faecal materials of corresponding infants (Martín et al., 2003). These studies simply conclude that breast milk contains LAB and bifidobacteria and a best source to function as initial inoculum for infants (Arboleya et al., 2012). Studies reveal that breast milk is a huge composition of diverse populace of bacteria such as Staphylococci, Streptococci, Micrococci, Lactobacilli, Enterococci, Lactococci and Bifidobacteria (Martín et al., 2004) and hence intake of breast milk is helpful in settling of bifidobacteria and lactobacilli in the infant gut. This have been proved that breast fed infant have lower susceptibility towards allergies as compared to formula fed infants. The lactobacillus strains isolated from breast milk activate natural killer (NK) cells and T cell subsets, which then lead to promotion of natural and acquired immune responses.

Human gastro intestinal tract (GIT) also a major source for probiotics. Previously explained that human gut houses more than 400 different species. Many probiotic strains used now have been isolated from human tract only which includes *L.gasseri* and *L. reuteri* (Ryan et al., 2008) including *L. fermentum* also. Studies show that the probiotic strains isolated from GIT

show potent characteristics as compared to isolated strains from other sources, among which antagonistic activity against the food borne pathogens is most important.

Many research studies reveal that non-dairy fermented and non-fermented food products are also a potent source for isolation of probiotic strains (Rivera-Espinoza and Gallardo-Navarro, 2010). From the in-vitro studies it has been found that some probiotic strains such as *L. sakei, L. curvatus* and *Staphylococcus carnosus* can be isolated from meats. Strains are also isolated from fruits such as *L. paracasei* and *L. plantarum* and these strains have same properties as that of isolated from human (Haller et al., 2001). Brines of naturally fermented Alorena green table olives were used and isolation of a lactobacillus strain was commenced. *L. buchneri* P2, which have been isolated from pickled juice have shown several probiotic properties including cholesterol reduction, acid and bile tolerance and antimicrobial activity (Zeng et al., 2010).

2.3.2 Isolation of probiotics

Before incubation in selective media the sample should be collected in adequate amount and it should be maintained. It has been found that most of the probiotics are aerobic and facultatively anaerobic, hence the sample should be kept in anaerobic conditions and then it should be processed quickly. For selective isolation of lactobacilli and bifidobacteria deMan Rogosa Sharpe (MRS) media is usually used which have been developed by Rogosa et al (Rogosa et al., 1951). MRS media acts as a selective media for the organisms because its low pH can be only tolerated by oral and faecal lactobacilli. The components of this media such as polysorbate 80 inhibit the growth of Gram-negative bacteria. The sample can be incubated in this media for 48-72 hours to get the full growth of the bacteria.

2.3.3 Identification of probiotics

Identification of the isolated bacteria is one of the steps for the selection of potential probiotics. The identification process involves both the genotypic and phenotypic approaches (Vandamme et al., 1996). Now-a-days 16S rRNA approach has become popular as this method has been used for microbiologists for last two decades for the phylogenetic classification of microbes (Winker and Woese, 1991). This approach has been combined with several other methods for better identification of microbes. The sequence coding for 16S rRNA is generally the 16S rDNA, which can be then amplified and coupled with PAGE using temperature, which is a temperature gradient gel electrophoresis or it can be subjected to

chemical denaturation also known as denaturing gradient gel electrophoresis (Muyzer and Smalla, 1998). Several other methods can also be used such as FISH (fluorescence in situ hybridisation) probes against specific to 16S rDNA (Langendijk et al., 1995). These DNA sequence can be further digested with restriction enzymes to employ Terminal restriction fragment length polymorphism (T-RFLP) for identification.

2.3.4 Characterization of isolates

In order to be used and to get its beneficial effects a potent strain must show some desirable characteristics. These are also known as selecting criteria. After passing these criteria a strain can be called as probiotic strain. Those criteria has been briefed in table 2 and they will be described later.

Table 2. Selection criteria for probiotics.

(Source: Çakır 2003)

Probiotic strain properties	Remarks
Human origin for human usage	Microorganisms from human gut will have more adaptability to live in human gut than from non-human sources.
Acid and bile tolerance	Important for oral consumption even if it may not be for other applications for survival through the intestine, maintaining adhesiveness and metabolic activity.
Adhesion to mucosal surface	Important to improve immune system, competition with pathogens, maintain metabolic activity, prevent pathogens to adhesion and colonization.
Safe for food and clinical use	Identification and characterization of strains should be done with accuracy and the safety assessment should be documented. No invasion and no degradation of intestinal mucus.
Clinically validated and documented health effects	Minimum effective dosage has to be known for each particular strain and in different products. Placebo controlled, double-blinded and randomized studies have to be run.
Good technological Properties	Survival in products if viable organisms are required, phage resistance, strain stability, culturable in large scales, oxygen resistance, have no negative effects on product flavour.

2.3.4.1 Low pH and bile salt tolerance

A potent probiotic strain must be acid tolerant because it ensures its viability and functionality in the gut where the pH is very low (Araya et al., 2002). Several in-vitro models or simulated gastric juices of pH 2.0-4.0 with incubation of 1-3 hours have been designed to evaluate the strains (Sanz, 2007). The strains can also be incubated in chemical or enzymatic media for 1-4 hours with pH of 1.5-3.0.

In GIT, the lipophilic compounds are digested by bile salts. The bile salts also act as an antimicrobial agent which helps to maintain homeostasis in the GIT. In human GIT the bile salt concentration is about 0.3-0.5% (Dunne et al., 2001). In vitro methods employs use of 0.3-0.7% of bovine bile (Oxgall) with incubation time of 1-3 hours.

Resistance to low pH and bile is both strain and species dependant. In several studies it has been found that bifidobacteria are highly sensitive to low pH with 0% survivability at pH 2 and incubation of 90 min (Charteris et al., 1998) whereas certain species have been found with 1% survival rate at pH 3.0 for 2 hours. Increased rate of survival was at pH 3.0-5.0 for 3 hours (Matsumoto et al., 2004). Where in case of lactobacillus strains show high resistance to low pH. Studies show that certain lactobacillus strains have survivability of 2-100% at pH 3.0 for 1 hour. Bifidobacteria have a survival rate of 1-70% against 0.3% Oxgall for 90 min. whereas in case of lactobacillus strains it has been found to be 3-70% (Bosch et al., 2012).

2.3.4.2 Adherence to intestinal epithelium

A potent probiotic strain must be able to adhere with the intestinal epithelial cell as well as to mucus because it will enhance the residence time of probiotics in the gut, competitive exclusion of pathogen and for host and immune system interactions. For the last 25 years Caco-2 cell line, a cancerous cell line has been under extensive use to study the adhesion capacity of probiotics (Dicks and Botes, 2010). The main cause of use of Caco-2 cell line is that, it forms a homogenous monolayer which mimics the human mature enterocytes present in the small intestine (Lenaerts et al., 2007); the formation of crypts by these cell line also resembles to that of intestinal epithelia (Huang et al., 2008). HT-29, a colonic cell line has been also used for in-vitro studies (Gopal et al., 2001).

Studies have shown that there is a difference in the adhesion of lactobacilli, bifidobacteria and pathogens to mucus, Caco-2, Caco-2 plus mucus, HT-29 MTX and Caco-2/HT-29 MTX.

For *L. rhamnosus* GG the adherence to the above systems have been found to be 10·21, 5·17, 3·19, 0·84 and 0·85 %, respectively (Huang et al., 2008).

2.3.4.3 Antimicrobial activity

This is one of the mechanism of action of probiotics by which they confer beneficial effects on host (Laparra and Sanz, 2009). Probiotics acts as antagonist to pathogen through variety of mechanisms such as production of antimicrobial substances, competition with pathogens for nutrients and adhesion sites and stimulation of the immune system (Collado et al., 2007). Variety of intestinal infections are result of binding of pathogens to intestinal mucosa which then leads to disruption of intestinal microbiota. Probiotics play an important role in protection against those pathogens (Sambuy et al., 2005).

Probiotics have shown antagonistic activity against *Listeria monocytogenes* and *Helicobacter pylori* (Chenoll et al., 2011) when tested in-vitro. They have also a promising effect against human rota-virus (Muñoz et al., 2011). Several strains of lactobacilli and bifidobacteria are able to successfully inhibit the growth of *Escherichia coli* (Gopal et al., 2001), *Salmonella typhimurium* (Jankowska et al., 2008), *Shigella flexneri* (Tien et al., 2006) and *C. difficile* (Pillai and Nelson, 2008). *L. plantarum* strain has been found to produce compounds with antifungal activity (Ryu et al., 2014).

2.4 Safety

Before use on human subjects or commercial utilization the safety assessment of probiotics should be evaluated. For this in 2002 in European Union "The European Food Safety Authority" was established to commence and set various guidelines as well as technical issues to check food and consumer safety under the regulation no. 178/2002. Unfortunately they have not assessed any guidelines for food associated microbes. An approach to assess the safety evaluation, was proposed by the Scientific Committee on Animal Nutrition, which is known as "Qualified presumption of safety" (Leuschner et al., 2010). This proposal has four steps for safety evaluation, which are as follows:-

1- Taxonomy of the microbe should be defined.
2- Sufficient information and data should be collected such as scientific literature, history of use, industrial application and ecological and human intervention data.
3- Exclusion of pathogenicity.
4- The end use should be defined.

For the successful commercialization of probiotics as food accessory or dietary supplement, safety evaluation of each particular strain for a general population must be performed (Sanders et al., 2010). In case of probiotics these following factors determine the safety evaluation:-

1- Proper collection and recording of isolation history and taxonomic classification of potent probiotics should be done.
2- Manufacturing should be done in controlled environment to eradicate the chances of cross contamination within batches of probiotics or with other microbes.
3- Assessment should be done at strain level to know the associativity of probiotics with infectivity or toxicity.
4- Dose administration and method of administration should be determined for every different population.

3. Objectives

The aim of this study was to identify the potent probiotics strain from a specific human population. The objectives of this study are as follows-

I- Isolation of indigenous probiotic strains from human faecal samples of Rourkela population.

II- Screening and characterization of strains with desired probiotic characteristics.

III- Identification of probiotics strains with indigenous potential.

4. Materials and Methods

4.1 Sample Collection and Isolation of Bacteria

4.1.1 Sample collection

Faecal samples were collected from healthy adult human being aged between 25-30 years and provided in sterile cilinicol by Ispat General Hospital (IGH), Rourkela.

4.1.2 Isolation of intestinal bacteria

About 1 gram of sample was taken through sterile spatula and then suspended into the MRS broth (de Man Rogosa Sharpe) supplied by HiMedia (Mumbai, India) and Cysteine-HCl media which is the anaerobic media for the isolation of anaerobic strains. The sample suspended in MRS broth was kept in incubator and the anaerobic media was kept inside gas-pack avoiding the contact with oxygen at 37^{o}C for 72 hours. Table 3 describes the composition of Cys-HCl media:-

Table 3. Composition of Cys-HCl media

Components	Weight in gm/1000 ml
Calcium Chloride	0.01
Glucose	10.0
Cysteine Hydrochloride	0.5
Magnesium Sulphate	0.008
Sodium Carbonate	4.0
Monobasic Potassium Phosphate	0.04
Peptone	5.0

Media became turbid due to growth of cells. The media was collected and centrifuged at 5000 rpm for 30 min. to collect the cell pellets. The cell pellets were them washed with 0.85% NaCl to remove any residual of media. Cell pellets were serially diluted in sterile NaCl solution and then spread plate was done on MRSA (MRS Agar) plates and kept for incubation at 37^{o}C for 24 hours. Colony forming units (CFU) appeared on plates then

morphologically different colonies were selected and to get pure culture they were streaked repeatedly as well as subcultured for further studies.

4.1.3 Acid and Bile treatment

As study on 155 isolates is not possible and there is a chance that some of these isolates may be the same, so the first hurdle to pass as a potent probiotic strain was performed. Initial screening of 155 isolates was done by treating them with various concentrations of acid and bile salt. MRS broth with pH of 3.0 was prepared and again MRS broth containing 0.5% of bile salt was prepared and the isolates were incubated in both media for 24 hours. After complete incubation cell pellet was collected and then serially diluted and spreading was done over MRSA plates. It was seen that lesser number of CFU have been appeared after incubation of plates. Based upon colony morphology different CFU were taken and cultured in microfuge tubes for further studies. Among these different isolates the survival rate was tested against differing concentration of acids and bile salt and best isolates were chosen for characterization.

4.2 Morphology and General Characters of Isolates

Simple tests such as Gram staining, Catalase test and Blood haemolysis test were performed for 4 best isolates.

4.2.1 Gram Staining

Gram staining is one of the novel technique for characterizing the bacteria as Gram-positive or Gram-negative. LAB found till now have known to be Gram-positive. Bacteria can be said as Gram-positive if it shows blue-purple colour after staining and if it shows pink colour then it is classified under Gram-negative.

The isolates were gown in freshly prepared media and incubated overnight. Fresh cultures were transferred to microfuge tubes and then centrifuged at 6000 rpm for 5 min and the supernatant was discarded. The cell pellet was collected and resuspended in sterile water. The cell were taken and then Gram staining procedure was followed. For viewing morphology light microscopy was done.

4.2.2 Catalase Test

Catalase activity or ability to produce catalase enzyme of microorganisms support them to live in aerobic environment. The enzyme breakdown the toxic hydrogen peroxide to oxygen and water. Anaerobic microbes lack the catalase activity. Catalase positive microbes produce bubbles when treated with H_2O_2 solution, as they release oxygen but the catalase negative don't. Catalase positive includes strictly aerobes and facultative anaerobes, whereas catalase negative microbes are strictly anaerobes.

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

Cultures were grown overnight on MRSA plates. The fresh cultures were taken with the help of sterile loop and smear was made on clear glass slide. On the smear 3% hydrogen peroxide solution was dropped and their catalase activity was observed.

4.2.3 Blood Haemolysis Test

As the strains were isolated from faecal material, blood haemolysis test was performed, to eradicate any chance that our isolates may be pathogenic. It is also one of the criteria for assessing the safety of use of probiotics as food supplements, when isolated from non-food products. Pathogens produce highly toxic substance which lyse the RBC and forms a clear zone around them. Isolates from the fermented food show no haemolytic activity.

Blood agar media is a composition of Tryptic Soy Agar and defibrinated blood either from sheep or human source. Table 4 shows the composition of the blood agar base:-

Table 4. Composition of blood agar base.

Components	Weight in gram/1000 ml
Pancreatic digest of casein	15.0
Papaic digest of soy meal	5.0
NaCl	5.0
Agar	15.0

After the preparation of media its pH was adjusted to 7.3 and then autoclaved. After sterilization the base was cooled to room temperature and then 5% defibrinated blood was added to the base avoiding the formation of bubbles. Then the media was poured over the

plate. Fresh cultures were taken with the help of sterile loop and streaking was done over the plates and kept for incubation. After complete incubation the haemolytic pattern was observed.

2.5 Screening and Characterization of Isolates

There are certain guidelines have been prepared by Indian Council of Medical Research, India to call a microbe as a probiotics. A microbe must fulfil these criteria to be classified as probiotics.

4.3.1 Acid and Bile Tolerance

This test has been performed earlier during isolation of potent strains.

4.3.2 Antimicrobial activity

Agar well plate method was followed to know the inhibitory effect of isolates against food borne pathogens. Four pathogens *E. coli, S. aureus, Salmonella typhimurium,* and *Enterococci* were used. These pathogens have been provided by Dr. Satish Sarkar from Sri Shakti Amma Institute of Biomedical Research, Vellore. These pathogens were cultured overnight and then swabbed on nutrient agar plates. Then wells were cut on the plates. About 50 µl of cell free supernatant from fresh cultures was taken and poured in the wells. Then the plates were kept for incubation for 24 hours undisturbed. After incubation the zone of inhibition was appeared and it was measured with the help of scale. For this test broth without inoculum was taken as control. *Lactobacillus casei Shirota* isolated from "Yakult" used as reference for our isolates.

4.3.3 Antibiotic Susceptibility Test

Probiotic strains must be sensitive towards the antibiotics. There is a high risk that antibiotic resistant probiotic strain may transfer the antibiotic resistance genes to the pathogen via transformation in the gut. This situation may lead to severe health complexity of a patient affected by bacterial infection and under treatment of antibiotics. Due to any chance events if such resistant pathogens get introduced into the human via food chain and cause serious problems.

Sensitivity of probiotic strains towards the antibiotics being tested by using Kirby-Bauer technique. Muller-Hinton agar plates were prepared then about 100µl of the fresh isolates were taken on the plates and swabbed all over the plates using a sterile cotton swab.

Antibiotic discs were then placed over the plates aseptically and then plates were left for incubation for 24 hours at 37^{o}C. After complete incubation the zone of inhibition was measured. Along with 4 isolates *L. casei Shirota* was used as reference.

4.3.4 Cell Surface Hydrophobicity Test

Cell surface hydrophobicity test is an actual determinant of bacterial adhesion to intestinal epithelia. This method has been used for various bacterial cell surface adhesion testing (Aswathy et al., 2008). About 10 ml of media was taken and strains were inoculated in it and left for incubation for 24 hours. Then the culture was taken and centrifuged at 6000 rpm for 5 mins and then washed with sterile NaCl solution. The cultures were resuspended in 10 ml of NaCl solution. The suspension was taken and absorbance (OD_A) was recorded at 600 nm. Then in 5 ml of cell suspension about 300 µl of apolar solvent, n-hexadecane was added and mixed fully by vortexing for 2 mins. The suspension was kept undisturbed and two phases were allowed to separate for 30 min and then again absorbance of aqueous phase (OD_B) was taken at 600 nm. Percentage of bacterial adhesion was measured by using this formula.

$$\% \text{ Bacteria adhesion} = [(OD_A - OD_B) \times 100]/OD_A$$

4.3.5 Simulated Gastric Fluid Tolerance

As name suggests simulated gastric fluid, mimics the gastric environment in the GIT. Survival of probiotics in GIT is essential to confer beneficial effects. Without proper molecular identification a strain can't be tested on human subjects. SGF provides an in-vitro environment same as the intestinal gastric fluid. The composition of SGF has been given in table 5.

Table 5. Composition of SGF

Components	Weight in gram/1000ml
Bile salts	0.085
Lecithin	0.051
Pepsin	0.1
NaCl	2.0

The pH of SGF was adjusted to 1.6 and then it was sterilized through membrane filtration method. Fresh isolates were taken and cell pellet was collected and then incubated with SGF for 2 hours. Then again culture was centrifuged and washed to collect cell pellets. These were

then serially diluted and then plated on MRSA plates. Normal untreated pellets were taken and plated as well. Plates were then incubated and enumeration was done.

4.3.6 Litmus Milk Assay

Litmus milk (HiMedia) is generally used for checking the action of lactobacilli on milk as well as maintenance of lactobacilli. *Lactobacillus* have a property to breakdown the lactose present in the milk and produce lactic acid. When lactobacilli is incubated in litmus milk, lactose present in it gets degrade and lactic acid is produced which changes the colour of media from blue to pink. As well as this assay also helps to know either the isolates are gas producing or not.

Litmus milk media was prepared and sterilized for 5 min. After cooling the media was transferred to small microfuge tubes. The tubes contained the fresh cell pellets of isolates. After inoculation it was placed for incubation and action of isolates on media was observed.

4.3.7 Phenol Resistance of Isolates

Spices are important ingredients in the Indian diet. Most of the used in Indian foods are found to be producing phenol on digestion in gut. Spices have shown their antimicrobial effect due to the production of phenolic compounds only. Aromatic amino acids either indigenously produced or taken in dietary uptake, gets deaminated in gut by bacteria and forms phenol. Phenols also show a bacteriostatic effect. Hence the newly isolated probiotic strains must be tolerant to phenol for their survival as well as health promoting effects.

Cultures were grown overnight and then 1% of culture was taken as inoculum for MRS broth and MRS broth added with 0.4% phenol. Then it was placed for incubation for 24 hours at 37^{o}C. After complete incubation cultures were grown on MRSA plates by spread plate technique. Finally enumeration was done to get the survival rate of isolates.

4.4 Special Features of Isolates

Isolates have been tested for some interesting features which are newly found in different probiotic strains. These features include Cholesterol reducing activity and Angiotensin Converting Enzyme-1 inhibitory activity.

4.4.1 Cholesterol Reducing Activity

About 1 ml of cell free supernatant of freshly cultured samples were collected but for blank no supernatant was taken. The volume was made up to 5 ml by adding $FeCl_3$-CH_3COOH reagent (0.05%). Then 3 ml of concentrated sulphuric acid was added. All the samples were incubated for 20 min at room temperature and then their absorbance was recorded at 560 nm (Zlatkis et al., 1953). This test was done by taking MRS media as sample because beef extract is an essential component of it and cholesterol has been found in beef extract.

4.4.2 ACE-I Inhibitory Activity

MRS broth was supplemented with 10% skim milk powder and was separately fermented by the 5 isolates for 48 hours. Then they were centrifuged at 7500 rpm for 20 min. The cell free supernatants were used as samples. The following assay protocol was followed. Absorbance was measured at 492 nm against blank (C). Table 6 describes the procedure for ACE inhibition.

Table 6. Procedure for assay of ACE inhibitory activity

Components	**A (sample)**	**B (control)**	**C (blank)**
100 mU/ml ACE (µl)	10	10	10
1M HCl (µl)	0	0	100
ACEI (sample) (µl)	20	0	20
100mM sodium borate buffer (µl)	0	20	0
Incubate at 37°C for 30 min			
5mM HHL (µl)	50	50	50
Incubate at 37°C for 30 min			
1M HCl (µl)	100	100	0
100mM sodium borate buffer (µl)	320	320	320
Quinoline (µl)	600	600	600
BSC (µl)	200	200	200
Incubate at 30°C for 30 min (in dark)			
Ethanol (µl)	3700	3700	3700
Incubate at 30°C for 30 min (in dark)			
Measure absorbance at 492nm			

ACEI% = [(OD Control- OD Sample)/ OD Control] x 100

4.4 Molecular Identification

The isolated strains are identified by 16S r-DNA sequencing of the highly conserved gene of 1.5 kb in length, 24F and 1492R primers are used to amplify the sequence by PCR. The sequencing was done using 27F and 785F primers and then merged to get a near full length sequence. The sequence was then taken and with the help of MEGA 4 phylogenetic tree was constructed to identify the strains.

3. Results and Discussion

5.1.1 Isolation of Potent Probiotic Strain

After complete incubation on broth and then by spread plate technique 155 isolates were selected based upon their different colony morphology then maintained for further testing.

5.2.1 Acid and Bile Tolerance

These 155 isolates upon treatment of low pH of 3.0 and bile salt of 0.5% these number get reduced to 10 isolates. Then again repetition of this test was done and best 4 isolates differing on morphology was selected. The isolates were designated as HIaeA, HIaeB, HIae5, HIae7.

5.2 General Characteristics of Isolates

5.2.1 Gram Staining

After gram staining of 4 best isolates, 3 of them found to be Gram positive bacillus and last one is the Gram positive and irregular rods.

5.2.2 Catalase Test

This test was done on 4 isolates along with the *L. casei Shirota*. Results show that HIaeA, HIaeB along with the *L. casei Shirota* were Catalase negative and HIae5, HIae7 were Catalase positive. The isolates showing negative results indicate that they are unable to produce catalase hence they can't degrade hydrogen peroxide. We can ultimately say that these catalase negative isolates were anaerobic or facultatively anaerobic. The isolates with positive results are strictly aerobic.

5.2.3 Blood Haemolysis Test

The patterns of the isolates were tested on blood agar. Isolates have grown on the plates without forming any clear zone. There haemolytic pattern as found to be "γ" which simply concludes that all of the isolates are non-haemolytic and belong to normal human microflora. These isolates were not capable to lyse the RBC of blood as well as not capable enough to degrade the proteins of blood serum. Figure 4 showing the γ haemolytic pattern of HIae 5 and HIae 7.

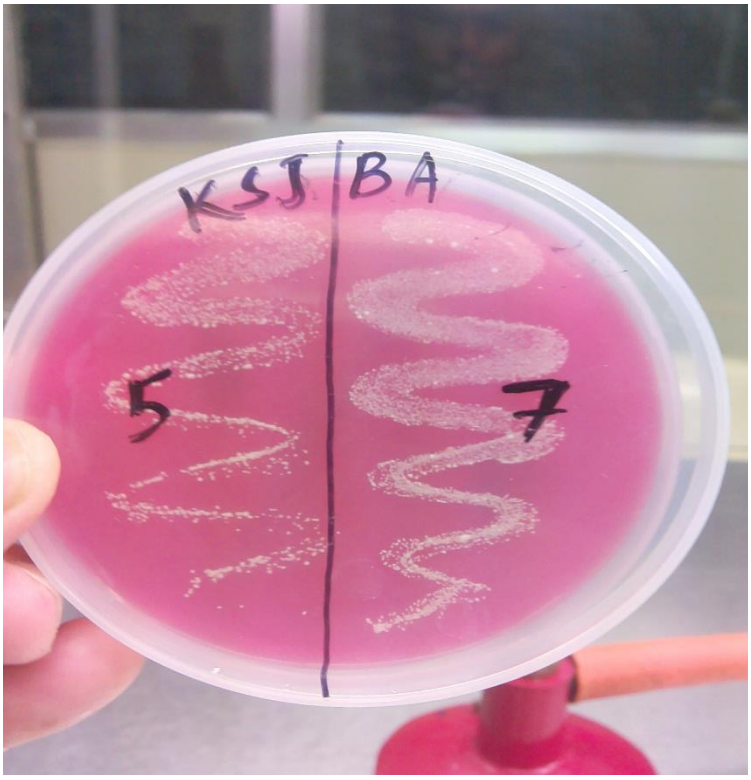

Fig 4:- γ haemolysis by HIae 5 and HIae 7.

5.3 Screening for Probiotic Characteristics

5.3.1 Antimicrobial Activity

Antimicrobial activity of isolates have been observed through their zone of inhibition. All of the isolates were found to be strongly inhibiting all of the food-borne pathogens. The highest zone of inhibition about 14.8 mm was found against *S. aureus* by HIae A strain. As compared to the previous studies done by other researchers, these results are more promising. Table 7 shows diameter of zone of inhibition.

Table 7. Antimicrobial activity of isolates against pathogens

Pathogens	Diameter of Zone of Inhibition in mm				
	L. casei Shirota	*HIae A*	*HIae B*	*HIae 5*	*HIae 7*
E. coli	10.66±0.47	10.33±0.47	12.65±0.73	10.7±0.31	8.33±0.31
S. aureus	10.7±0.94	14.8±0.48	11.56±0.48	10.66±0.57	11.66±0.47
Salmonella typhi	14±0.81	11±0.81	10±0.81	11±0.21	11.34±0.79
Enteococci	11.67±0.47	11±0.81	10.67±0.79	11±0.43	13±0.94

Probiotics have an inbuilt antimicrobial activity against many pathogens and this is due to mainly the oxidative properties which leads to change in the cell membrane of pathogens. Hence probiotics are promising than other antibiotics used for curing the bacterial infections. It has been known that LAB strains are capable of producing organic acids, hydrogen peroxide, diacetyl compounds, lactobiocin a bacteriocin and several bactericidal proteins during lactic acid fermentation. These strains can be used against the following diseases Hemorrhagic Colitis caused by *E. coli*; Staphylococal intoxication caused by *S. aureus* and Salmonellosis caused by *S. typhimurium*.

5.3.1 Antibiotic Susceptibility Test

Isolates have shown varying pattern of susceptibility against six different antibiotics tested. Three isolates have been found to be resistant to methicillin. Highest sensitivity found against gentamicin by HIae 7 about 40 mm in diameter, whereas the lowest was about 11 cm against streptomycin by HIae A. Table 8 shows the diameter of zone of inhibition by antibiotics.

Table 8. Antibiotic susceptibility of isolates test using Kirby-Bauer method

Antibiotic Disks	Diameter of Zone of Inhibition in mm				
	L.casei Shirota	*Hiae A*	*HIae B*	*HIae 5*	*HIae 7*
Streptomycin	28.66±0.94*	11.63±0.95$^{\#}$	26±0.81*	35.66±0.94*	28.33±1.24*
Tetracycline	38±0.72*	24.32±1.56*	26.52±1.05*	20±1.63*	19.66±1.35*
Gentamicin	36.66±1.69*	16±1.62*	39±0.81*	38.66±0.94*	39±0.81*
Penicilin	38.66±1.24*	28.33±0.88*	29±1.32*	36±0.85*	32.33±1.67*
Erythromycin	37.85±1.21*	29.64±1.13*	26.66±1.21*	38±0.91*	20.33±0.73*
Methicilin	12±0.81*	14±0.81*	0	0	0

Moderately sensitive-*; Highly sensitive-$^{\#}$; Resistant-0

The isolates which are found to be methicillin resistant but they are sensitive towards penicillin. Both of the antibiotics share same mechanism of action by inhibiting the transpeptidation enzymes involved in cross-linking of D-ala-D-ala subunits in cell wall synthesis. Methicillin is a narrow spectrum antibiotic whereas penicillin is known to be a broad spectrum antibiotic.

5.3.2 Cell Surface Hydrophobicity

Cell surface hydrophobicity was found to be highest for HIae 5 and lowest for the *Lactobacillus casei Shirota.* Bacterial cell surface has a lipid layer integrated with polysaccharides such as NAM and NAG, and they form a lipopolysaccharide layer at their cell wall. Their attachment towards a non-polar solvent concludes the possibility of their attachment to the intestinal epithelia. Studies show that hydrophobicity of cell surface of a bacteria is directly proportional to level of adhesion (Rijnaarts et al., 1993). Graph 1 shows the percentage of hydrophobicity of strains towards n-hexadecane.

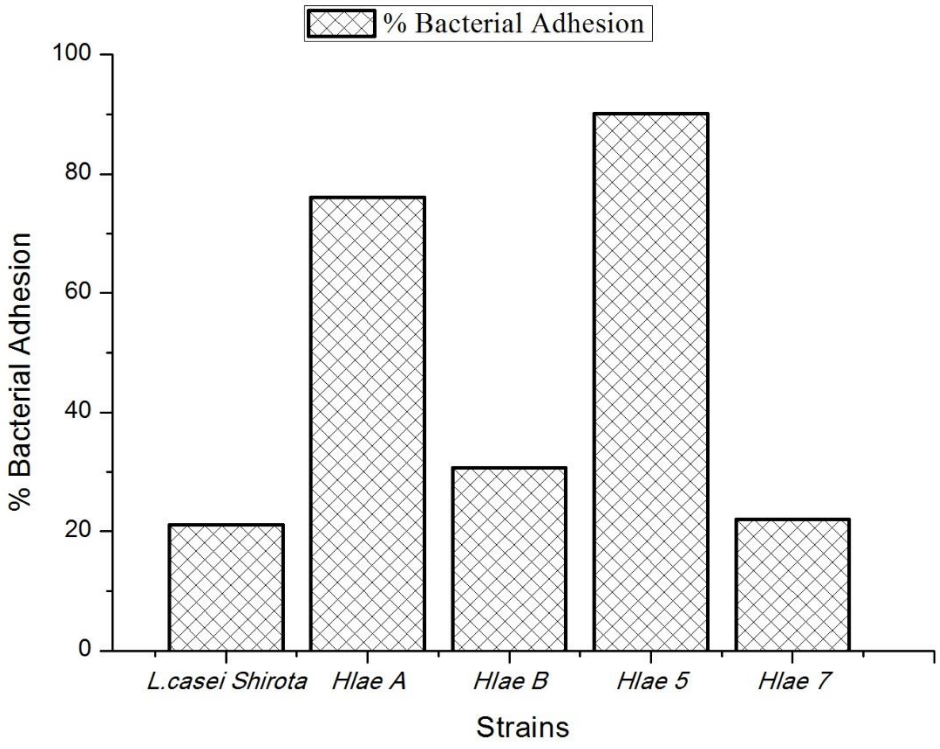

Figure 5. Adhesion property of human isolates.

5.3.4 Litmus Milk Assay

All of the isolates incubated in litmus milk changed the colour of the media from blue to pink as an indication of acidic reaction. Lactose present in the milk of media broken down to lactic acid, which reduced the pH of media and change in the colour was observed. Among the isolates HIae 5 and HIae 7 found to be gas producing.

5.3.3 Phenol Resistance

After incubation with 0.4% phenol for 24 hours, the survival rate of the isolated didn't change too much. Highest survivability about 99.46% is seen in strain HIae 5 and the lowest 98.76% for HIae 7. The higher survival rate of isolates indicate them as a novel probiotic strain. There was no significant change found in the growth of isolates even after treatment with phenols. Figure 6 showing the rate of survivability of treated cells.

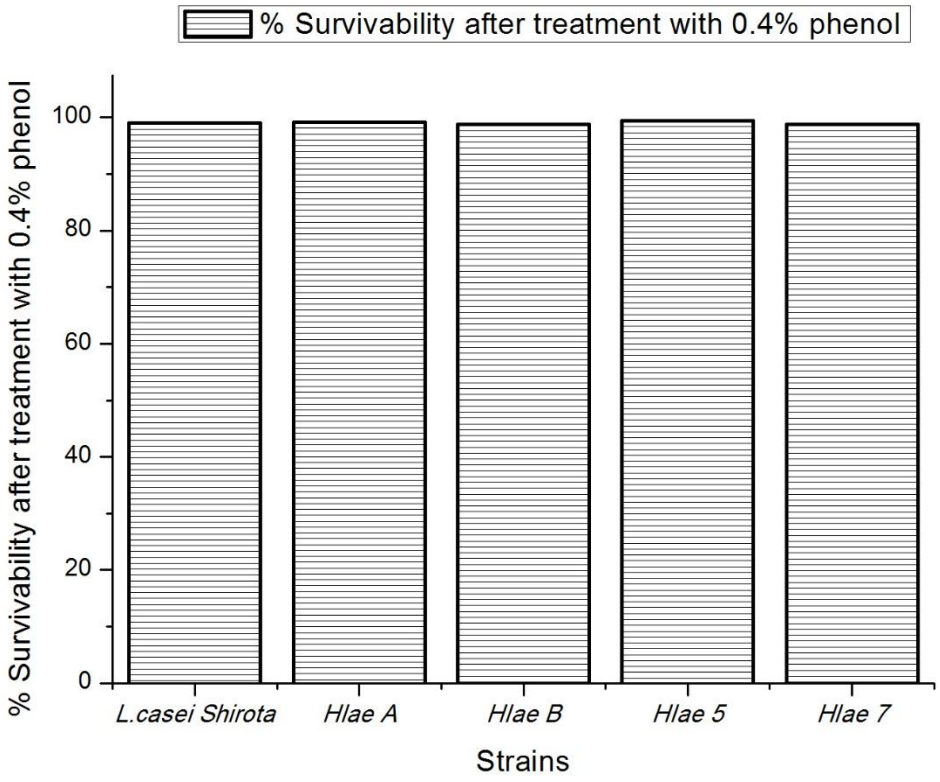

Figure 6. Survivability of isolates after treatment with 0.4% phenol.

5.3.4 Simulated Gastric Fluid (SGF) Tolerance

It was observed that there is negligible change in the number of surviving cells after treatment with SGF. After incubation highest rate of survival was 99.39% found for HIae A strain, whereas the lowest was 98.72% for HIae B. Survival through simulated gastric fluid depends upon the bile salt hydrolase activity, survival in low pH as well as NaCl. These three components of gastric fluid exert their effects on the survival of microbes in GIT. However this much high rate of survivability through SGF indicates that these strains are potent probiotics. Graph 3 showing the % survival rate of cell after SGF treatment.

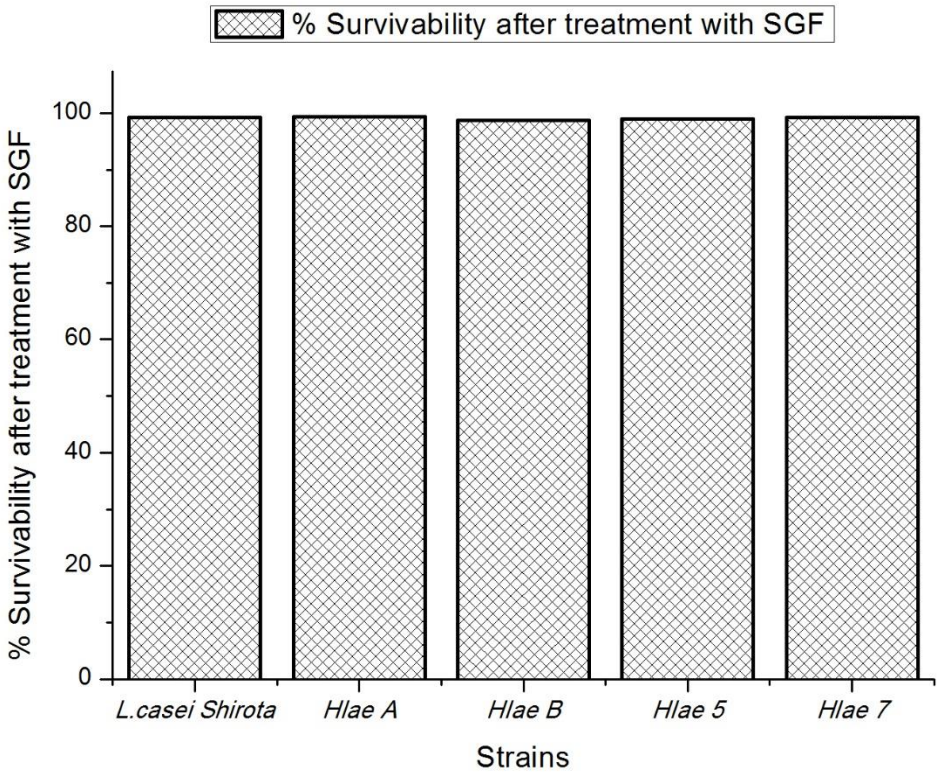

Figure 7. Survivability of isolates after treatment with SGF for 2 hours.

5.4 Special Features of Isolates

5.4.1 Cholesterol Reduction Capability

One of the main reason for cardiovascular disease is the high level of cholesterol in blood serum. To reduce the chance of getting cardiovascular disease, the blood cholesterol level should be brought down. In many studies probiotic strains have shown to be reducing the cholesterol. Among the tested isolates highest cholesterol reduction was 55.67% for strain HIae B and lowest was 49.38% for *L.casei Shirota.* Cholesterol reducing capability of the four isolates are same with the variance of only 1%, but these are higher than the L.casei Shirota. Hence regular administration of these four isolates will be helpful in lowering of blood cholesterol level. Figure 8 showing the capability of cholesterol reduction by the isolates.

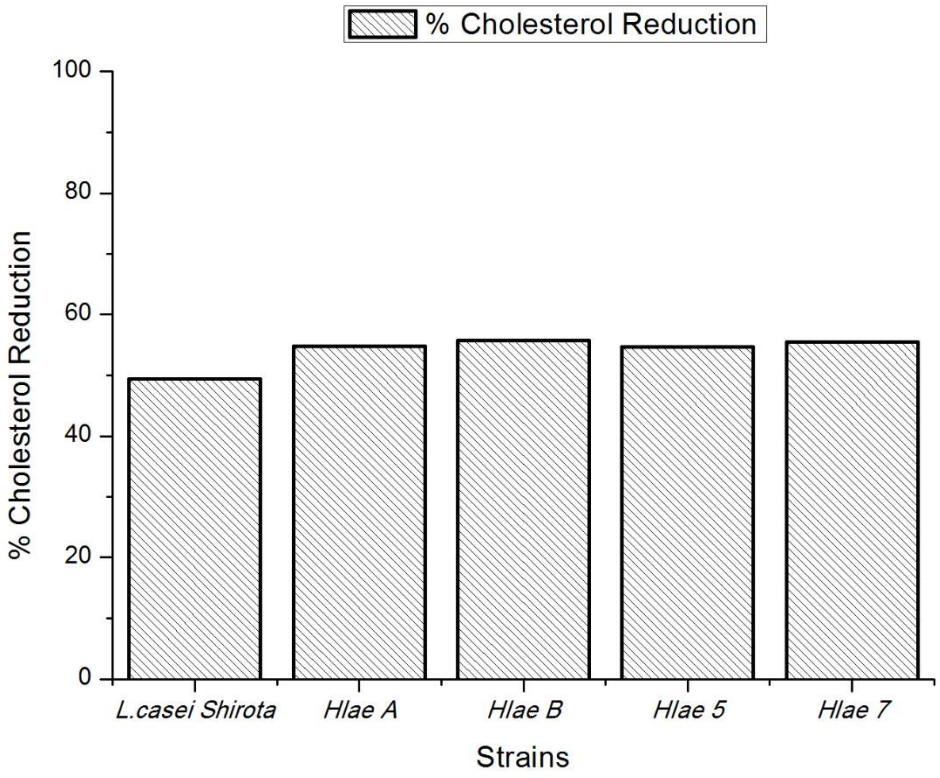

Figure 8. Cholesterol reduction capability of isolates.

It has been found in studies that lactobacilli upon growth causes breakdown and precipitation of cholesterol with the lowering pH as well as bile salts in a repeated manner which further disrupt the structure of cholesterol micelles present in the media, which one of the best reason of cholesterol removal by probiotics.

5.4.2 ACE Inhibitory Activity

Among the isolates tested, HIae B found to be causing maximum inhibition of 79.4% and minimum was 5.9% by *L. casei Shirota*. Figure 8 showing the Angiotensin Converting Enzyme inhibition by isolates.

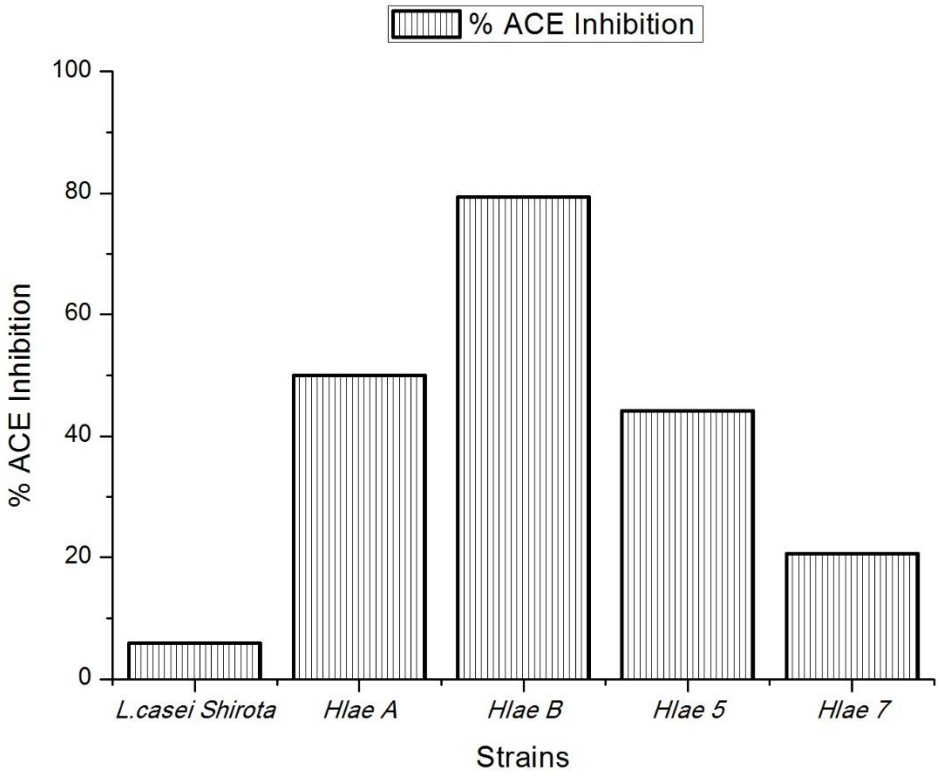

Figure 9. ACE inhibitory activity of isolates

During incubation with skim milk present in the media, isolates lead to several biochemical functions along with the lactic acid and flavour compounds. The most important biochemical reaction is proteolysis due to which decomposition of casein present in milk occurs resulting in several oligopeptides. Some of these oligopeptides lead to release specific amino acid sequences considered as bioactive peptides due to their physiological effects. Formation of bioactive peptide is solely dependent on the proteolytic activity of the isolates.

Probiotics generate ACE inhibitor peptides having a proline residue at the carboxy terminal end. Proline has capability to escape from degradation by many digestive enzymes and in the

form of short peptides it can pass through small intestine to blood circulation, where it exerts its beneficial affect by inhibiting the ACE.

5.5 Molecular Identification

DNA sequence was obtained for HIae 5 and HIae 7 out of four isolates by 16S r-DNA sequencing. The sequences were then put in blastn to obtain the sequences with higher similarity. Through MEGA 4 phylogenetic trees were created. HIae 5 was found to be *Lactobacillus plantarum* and HIae 7 was found to be *Weisella confusa*. Figure 10 and 11 showing the phylogenetic tree of strains.

16S rDNA partial sequence of isolates:-

HIae 5-

TAATGCAGTCGACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTG
AGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGG
ATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCC
GAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGC
TAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGG
GTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTG
AAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAG
TAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAA
AGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAA
GAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCC
ATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGG
CTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGAT
TAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTT
CCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCC
GCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCA
AATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAA

CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA
CACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTA
ATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAG
TCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGG
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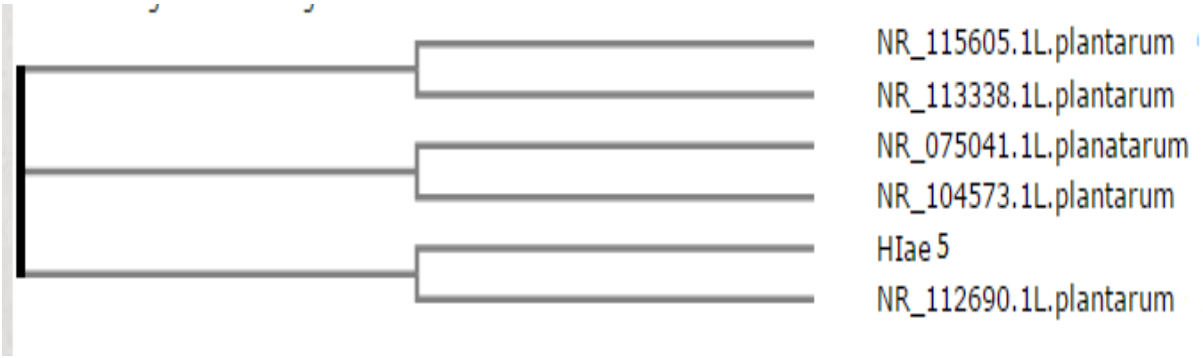

Figure 10. Phylogenetic tree of HIae 5.

HIae 7-

GCNANAATGCAGTCGANGCTTTGTGGTTCAACTGATTTGAAGAGCTTGCTCAGAT
ATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAAC
CTACCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAAT
GACAACCGCATGGTTGTTATTTAAAAGATGGTTCTGCTATCACTAAGAGATGGTC
CCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGATGATGCAT
AGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGC
AACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGAGAA
GAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAA
CGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGG
ATTTATTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCC
CTCAGCTCAACTGAGGAATTGCTTTGGAAACTGGATGACTTGAGTGCAGTAGAG
GAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACC
AGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGG
GTAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAGTGCTA
GGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCTAACGCATTAAGCACTCCGC

CTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTC
TTGACATCCCTTGACAACTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCC
GCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGATTGTAGGCTGCAAC
TCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACAC
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TTAGGGTGAAGTCGTAACAAGGTAAACCGTA

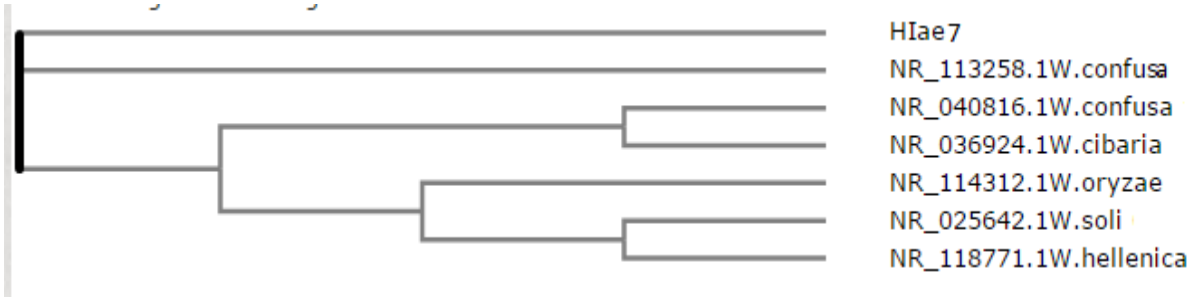

Figure 11. Phylogenetic tree of HIae 7.

6. Conclusion

Isolation, characterization for probiotic properties and identification of strains form faecal samples of a specific population of Rourkela were the objectives of this study. Isolates were tested all predetermined probiotic properties such as low pH and bile tolerance, antimicrobial activity, susceptibility to antibiotics, cell surface hydrophobicity, resistance to phenol and simulated gastric fluid etc. Then these isolates were again tested for two additional characters i.e. cholesterol reduction and ACE inhibitory activity. After passing all potential probiotic properties these isolates were identified by 16S rDNA sequencing. Final results are as follows:-

1) Potential probiotic strains were isolated from faecal material.
2) Only four isolates showed best resistance against acid and bile tolerance and then used for further study.
3) Best four isolates along with *L. casei Shirota* of commercial fame were tested for many potential probiotic characters. Our isolates showed better results in comparison to *L. casei Shirota* in tests like cell surface hydrophobicity, cholesterol reduction, ACE inhibition etc. Our isolates also showed higher antagonism to certain pathogens in comparison to *L. casei Shirota.*
4) Upon genotypic level of identification two of our isolates HIae 5 and HIae 7 were found to be *L. plantarum* and *W. confusa*.

7. Future Perspectives

A potent probiotic strain after identification and prior characterization and approval of safety it must go through the clinical evaluation to know whether these isolates are effective or not. To know the beneficial effects of our isolates studies like randomised, placebo-controlled trials can be done.

Then for commercialization and for industrial applications study on two most important aspects can be done. These aspects are i) adequate medium enhancing the growth of probiotics in large quantity and ii) cell viability during manufacturing as well as shelf life of probiotics during storage. However to increase cell viability and shelf life many procedures are available such as encapsulation, freeze drying etc. These studies can also be done on our isolates.

It has been known that probiotics modulate innate and adaptive immune system of human, but still the molecular basis of these effects are still not known.

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